# Purification of Lipoxygenase from *Chlorella*: Production of 9- and 13-Hydroperoxide Derivatives of Linoleic Acid

**Alberto Nuñez*, Brett J. Savary, Thomas A. Foglia, and George J. Piazza**

USDA, ARS, ERRC, Wyndmoor, Pennsylvania 19038

**ABSTRACT:** Oxygenation of linoleic acid by the enzyme lipoxygenase (LOX) that is present in the microalga *Chlorella pyrenoidosa* is known to produce the corresponding 9- and 13-hydroperoxide derivatives of linoleic acid (9- and 13-HPOD, respectively). Previous work with this microalga indicated that partially purified LOX, present in the 30–45 and 45–80% saturated $(NH_4)_2SO_4$ precipitate fractions, produced both HPOD isomers but in different ratios. It was not clear, however, if the observed activity in the two isolates represented the presence of one or more isozymes. In the present work, LOX isolated from the intracellular fraction of *Chlorella* by $(NH_4)_2SO_4$ precipitation (35–80% saturated) was purified by ion exchange and hydrophobic interaction chromatography to apparent homogeneity. Analysis of the purified protein by SDS-PAGE and subsequent native size exclusion chromatography demonstrated that LOX in *Chlorella* is a single monomeric protein with a molecular mass of approximately 47 kDa. The purified LOX produced both the 9-HPOD and 13-HPOD isomers from linoleic acid in equal amounts, and the isomer ratio was not altered over the pH range of 6 to 9. Optimal activity of LOX was at pH 7.5.

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Lipoxygenase (LOX; EC 1.13.11.12) catalyzes the addition of molecular oxygen to PUFA to form hydroperoxide derivatives. This enzyme promotes the regio- and stereoselective dioxygenation of PUFA containing one or more 1(*Z*),4(*Z*)-pentadiene bond systems to produce (*Z*,*E*)-conjugated monohydroperoxy FA (1,2). LOX is widely distributed throughout the plant and animal kingdom and can be classified according to its specificity. In higher plants, LOX is classified according to its specificity on linoleic acid (LA) or linolenic acids. The enzyme is characterized as 9-LOX when the main product formed is 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPOD) or 13-LOX when 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid isomer (13-HPOD) is the product (1,3). However, other minor amounts of positional isomers also can be formed. For example, soybean LOX, which produces 13-HPOD as the main product, also produces small amounts of 9-HPOD (1). In higher plants, additional forms of the enzyme produce a mixture of HPOD isomers (1–3).

LOX activity also is found in microorganisms such as fungi (4) and microalgae (5–7). Zimmerman and Vick reported LOX activity in the single-cell microalga *Chlorella pyrenoidosa* that produced 13-HPOD as the predominant oxidation product of LA. This activity was present in the fraction from the crude intracellular extract that precipitated from 0–42% saturated $(NH_4)_2SO_4$. This LOX was reported to have a maximum activity at pH 7.5 (5) and an apparent M.W. of 182 kDa (8). Bisakowski and Kermasha later reported that *C. pyrenoidosa* also contained a LOX activity in the fraction precipitated from 40–80% $(NH_4)_2SO_4$ (7). They reported that this LOX showed optimal activity at pH 4.5. Nondenaturing gel electrophoresis of this LOX-containing fraction indicated a M.W. range between 67 and 140 kDa, but product specificity was not reported. Subsequent work reported the 9-, 10-, and 13-HPOD isomers were formed from LA using a LOX fraction precipitated from 40–80% $(NH_4)_2SO_4$ at pH 7.0 (9). These studies, however, did not establish whether the reported LOX activities were the result of one or more isozymes.

Recently we reported that in addition to its peroxidation activity, LOX present in *C. pyrenoidosa* also cleaved 13-HPOD to a $C_5$ fragment and a $C_{13}$ oxo-FA under anaerobic conditions (10). The LOX fraction precipitated from 40–80% $(NH_4)_2SO_4$ was further purified by size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC). LOX peroxidation and the anaerobic cleavage activity eluted coincidentally with these chromatographic purification techniques, as similarly reported for soybean LOX (11,12), but the LOX peroxidation products were not characterized (10).

In a following study, we reported an HPLC method with EI-MS detection (LC/EI-MS) that allowed the analysis of the methylated HPOD products formed by LOX. This method does not require derivatization of the hydroperoxy group as required by GC with mass detection (GC–MS) (13). Using the LC/EI-MS method, we analyzed the products formed by LOX present in the *C. pyrenoidosa*-derived fraction precipitated from 30–45 and 45–80% $(NH_4)_2SO_4$. The 30–45% $(NH_4)_2SO_4$ fraction gave an HPOD product distribution that was dominated by the 13-HPOD isomer, consistent with the results reported by Zimmerman and Vick (5). The 45–80% fraction, however, contained primarily the 9-HPOD isomer, and the 10-HPOD isomer (10-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid) reported by Bisakowski *et al.* (9) was

*To whom correspondence should be addressed at USDA, ARS, ERRC, Fats, Oils and Animal Coproducts Unit, 600 East Mermaid Lane, Wyndmoor, PA 19038. E-mail: anunez@arserrc.gov

Abbreviations: HIC, hydrophobic interaction chromatography; 9-HPOD, 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 10-HPOD, 10-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid; 12-HPOD, 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid; 13-HPOD, 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; IEC, ion exchange chromatography; LA, linoleic acid; LOX, lipoxygenase; MWCO, molecular weight cutoff; SEC, size exclusion chromatography.

not found. These results suggested the presence of two LOX isozymes.

In this study, the LOX activity present in the microalga *Chlorella* was purified to apparent electrophoretic homogeneity. The HPOD products formed from LA using this purified LOX were analyzed by LC/EI-MS to clarify previous reports that suggested the presence of LOX isozymes.

## MATERIALS AND METHODS

*Materials.* LA was purchased from Sigma Chemical (St. Louis, MO). All other reagents used were of the highest purity available. Products were methylated with diazomethane before LC/EI-MS analysis (13).

*Algae growth and protein extraction. Chlorella pyrenoidosa* from American Type Culture Collection (no. 11469; Manassas, VA) or its equivalent, *C. fusca,* from the Cultural Collection at UTEX (no. 251; Austin, TX) was grown, harvested, and processed for protein extraction as described previously (14). Soluble proteins from the crude extract were partially purified by $(NH_4)_2SO_4$ precipitation at 0°C. The 0–35% fraction, containing most of the chlorophyll pigments and negligible LOX activity, was discarded and the 35–80% fraction used for further purification. Procedures for these steps as well as protein concentration assay were reported (13).

*Chromatographic protein purification.* Proteins present in the fraction precipitated from 35–80% saturated $(NH_4)_2SO_4$ were dissolved in 5 mL of Tris buffer (10 mM, pH 8.0) and dialyzed with a 14,000 M.W. cutoff (MWCO) Spectra/Por membrane (Spectrum Laboratories, Rancho Dominguez, CA) against 4 L of buffer. Chromatography was performed on a BioCAD 700E system with Poros perfusion media (PerSeptive Biosystem, Inc., Framingham, MA). LOX activity was first separated on a Poros-HQ20 anion-exchange chromatography column (10 mm diameter × 100 mm length, column volume 7.9 mL) using Tris buffer (10 mM, pH 8.0) at 5 column vol/min with a linear gradient of 30 column vol (about 240 mL) to a final concentration 0.20 M NaCl, in the same buffer. Fractions of 0.5 column vol (*ca.* 4.0 mL) were collected and assayed for LOX activity. The active fractions were pooled and concentrated with a Centricon-10 membrane filter (Amicon, Beverly, MA); then $(NH_4)_2SO_4$ was added to a final 2.0 M. The sample was then loaded onto a Poros-PE20 hydrophobic interaction chromatography column (100 × 4.6 mm, column vol. 1.7 mL). The column was equilibrated with 2.0 M ammonium sulfate in Tris buffer, then developed with a linear gradient of 25 column vol (42 mL) to 0.0 M ammonium sulfate (100% 10 mM Tris buffer, pH 8.0) at a flow rate of *ca.* 5 mL/min. Fractions of 0.5 column vol were collected and assayed for LOX activity. The active fractions were pooled and concentrated for electrophoretic analysis and incubated with LA to determine product specificity.

Native determination of LOX M.W. involved treating the $(NH_4)_2SO_4$-treated *Chlorella* extract by dialysis using Spectrum CE DispoDialyzer (Spectrum Laboratories) 50,000 MWCO dialysis tubes with Tris buffer (10 mM, pH 8.0). The dialyzed fraction was passed (five times) through a DE Sepra-Sorb 10-mL cartridge (Sepragen Corporation, Hayward, CA) that had previously been equilibrated with Tris buffer (10 mM, pH 8.0) containing NaCl (0.1 M). The unbound proteins containing the LOX activity (final volume of 10 mL) were concentrated and further resolved by SEC with a High Prep 26/60 Sephacryl S-200 column (Pharmacia Bitoech Inc., Alameda, CA). Fractions of 5.6 mL were collected and assayed for LOX activity. The active fractions were combined, concentrated, and incubated with LA for product analysis by LC/EI-MS. Final M.W. estimation of the LOX activity eluted from the Sephacryl S-200 column was performed with a Bio-Silect SEC 125-5 column (Bio-Rad, Hercules, CA). The column was run with potassium phosphate (200 mM, pH 8.0) at 1 mL/min using a Waters Separation Module 2690 (Waters Co., Milford, MA); fractions were collected and assayed for LOX activity. The Sephacryl-S200 and the Bio-Silect SEC columns were calibrated using the following M.W. standards: glucose-oxidase (120,000), BSA (67,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700).

*Electrophoresis.* Proteins were resolved by SDS-PAGE using a 12% gel in a Mini-Protein electrophoresis cell following the manufacturer's instructions (Bio-Rad). Samples were reduced using 25 mM DTT. Molecular sizes were calculated with Bio-Rad broad range M.W. marker proteins (200,000, 116,000, 97,400, 66,200, 45,000, 31,000, 21,500, 14,400, and 6,500). About 5 to 15 µg total protein was loaded per lane, and gels were stained with Coomassie brilliant-blue R-250.

*LOX activity assay and product characterization.* Enzymatic activity was assayed by adding 5–20 µL of the enzyme concentrate to a solution of 900 µL LA (1 mM) in potassium phosphate (100 mM, pH 8.0) and Tween 20 (1%) to final volume of 1 mL and by monitoring the initial rate of increase in absorbance at 234 nm.

For product characterization, active LOX fractions were incubated with 50 mL of the LA solution under oxygen, and the progress of the reaction was followed spectrophotometrically at 234 nm. At the end of the reaction the products were extracted with ether, methylated, and analyzed by LC/EI-MS. LC was performed with a Waters HPLC 2690 Separation Module connected in series to a Waters 996 Photodiode Array Detector and a Waters Thermabeam Mass Detector (Integrity System). The LC portion used a Valco LiChrosorb Diol 5 µm column (2 × 250 mm) (Varian/Chrompack, Raritan, NJ) using the method reported previously (13).

## RESULTS AND DISCUSSION

*Purification of LOX activity.* Previously we reported that the fractions obtained from the protein extract of *C. pyrenoidosa* and precipitated from 45 and 80% saturated $(NH_4)_2SO_4$ had LOX activity that produced the 9- and 13-HPOD isomers, but in different ratios (13). These results suggested the presence of two LOX isozymes, which was consistent with other reports on LOX activity in the microalga (5,9). In this study the

microalga *C. fusca* was used as an alternative source of *C. pyrenoidosa*, since it was found before that these two strains have the same enzymatic activity associated with LOX (10,14,15). Product characterization of LOX for both strains in the 30–45 and 45–80% fractions showed similar product distributions (see below). In addition, the *C. fusca* is offered as equivalent to *C. pyrenoidosa* by UTEX.

To determine the presence of one or more isozymes in *Chlorella*, LOX activity expressed in the fraction precipitated from 35–80% $(NH_4)_2SO_4$ was sequentially purified by ion exchange chromatography (IEC) and HIC. Figure 1A shows the separation of the dialyzed $(NH_4)_2SO_4$ fraction using a Poros-HQ20 anion exchange column with protein detection at 280 nm. LOX activity eluted under the well-defined peak noted in Figure 1A, indicating the weak binding of this protein to this column in relation to the other proteins present in the 35–80% fraction. Since in a previous report the LOX activity precipitated at different percentages of $(NH_4)_2SO_4$ seemed to determine the ratio of the HPOD isomers formed (10), the active fractions from this anion exchange column were concentrated and treated with 2 M $(NH_4)_2SO_4$ and further resolved using HIC with a Poros-PE20 column. Figure 1B shows the HIC chromatogram with detection at 280 nm, where a single LOX activity eluted under the later peak as indicated. Table 1 summarizes the purification steps, indicating a 1.2-fold increase in the specific activity after $(NH_4)_2SO_4$ precipitation and a 270-fold increase after IEC. Despite the removal of other proteins by HIC, however, specific activity decreased about 50% (Table 1). This significant total activity loss was associated with the concentration step of the IEC fraction after ultrafiltration at 10,000 MWCO. The reason for this loss in LOX activity is not clear and needs to be further studied. On the other hand, the elution of the LOX activity from the Poros-PE20 column was consistent with our early report of LOX activity in *C. pyrenoidosa*, in which the peroxidation and anaerobic cleavage activity eluted under the same conditions (10).

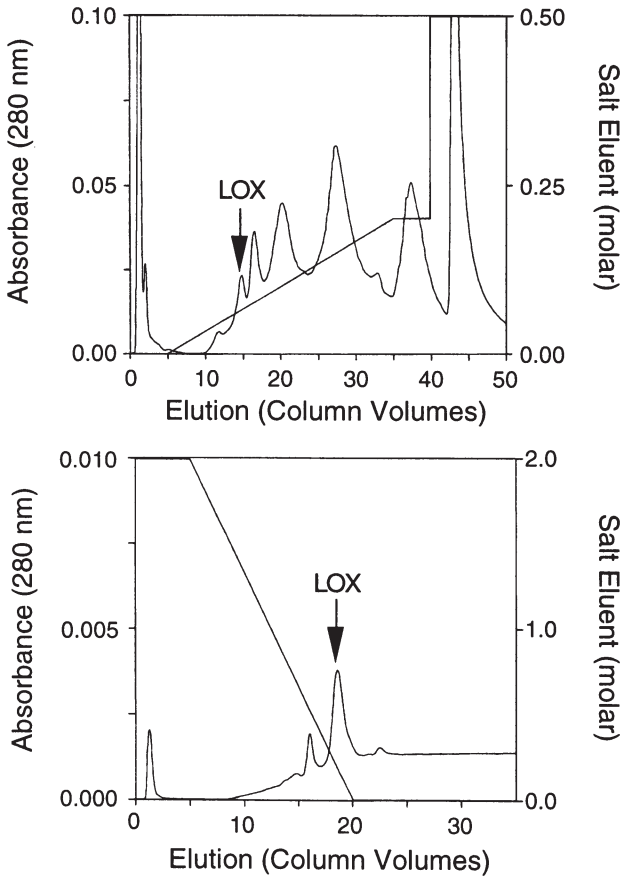


**FIG. 1**. Sequential adsorption chromatography purification of *Chlorella* fraction precipitated from 35–80% $(NH_4)_2SO_4$. Position of lipoxygenase (LOX) activity peaks is indicated by arrows. (A) Primary separation on Poros-HQ anion-exchange column (PerSeptive Biosystems, Inc., Framingham, MA). (B) Subsequent separation by hydrophobic interaction on Poros-HP2 column. One column-volume fractions were collected and assayed for LOX activities.

Figure 2A shows the SDS-PAGE of the purified LOX fractions after the two-mode adsorption chromatography sequence. The electrophoretic pattern indicated two major proteins in an approximate 1:1 ratio, each with an estimated M.W. of 34 and 47 kDa, respectively, but did not provide direct information on the native size of the LOX activity.

*Size exclusion purification*. SEC was used to determine the native size of LOX. The 35–80% $(NH_4)_2SO_4$ fraction was dialyzed against a 50,000 MWCO membrane and then passed through a DEAE ion-exchange cartridge using conditions where the protein having LOX activity did not bind to the ion-exchange support (see the Materials and Methods section). The protein with LOX activity was purified further by SEC on a Sephacryl 200 column. The SEC protein fractions eluting from the column were assayed for LOX activity. The results are plotted in Figure 3A, which shows that the enzymatic

**TABLE 1**
**Purification of Lipoxygenase from *Chlorella* by Absorption Chromatography[a]**

| Stage | Total protein (mg) | Total activity (μmol/min) | Specific activity (μmol $min^{-1}$ $mg^{-1}$) | Yield (%) | Purific. (fold) |
|---|---|---|---|---|---|
| Crude extract | 60.1 | 96.3 | 1.6 | 100 | 1 |
| $(NH_4)_2SO_4$ | 50.7 | 91.2 | 1.8 | 94.7 | 1.2 |
| Dialysis (14 K)[b] | 47.1 | 82.6 | 1.7 | 85.8 | 1.1 |
| IEC | 0.095 | 38.6 | 405 | 40.1 | 270 |
| HIC | 0.025 | 4.9 | 196 | 5.1 | 131 |

[a]Abbreviations: Purific., purification; IEC, ion exchange chromatography; HIC, hydrophobic interaction chromatography.
[b]Cutoff of 14,000 M.W.

LOX activity peaks between the 67 and 25 kDa M.W. markers. Fractions 24–30 containing the LOX activity were pooled and concentrated. SDS-PAGE analysis of the later fractions indicated the absence of the 34 kDa protein (Fig. 2A) and the presence of an intense band at 47 kDa, as shown in Figure 2B. The active pooled fractions from the Sephacryl column were resolved using a high-resolution Bio-Silect SEC 125-5 column. Fractions from this column were collected and assayed for LOX activity, and the results are shown in Figure 3B, thus confirming that LOX activity corresponded to a protein with a M.W. of approximately 47 kDa. These results show that LOX activity in *Chlorella* is due to a monomeric enzyme of 47 kDa size, and that the smaller 34 kDa protein (Fig. 2A), which is isolated in the adsorption chromatography steps, does not have LOX activity. The native size determined for LOX, however, is not consistent with the previously reported M.W. of 182 and 120 kDa in *C. pyrenoidosa* (8,10). We have in fact observed a minor LOX activity in the $(NH_4)_2SO_4$ fraction eluted from the Sephacryl 200 column at the previously reported size of 120 kDa, Figure 3A (10), but its activity was lost in the subsequent purification process. Analysis of the products formed by the 120 kDa LOX from the Sephacryl column were not different from the products formed by the 47 kDa LOX, suggesting the possible aggregation of LOX protein in the early purification steps.

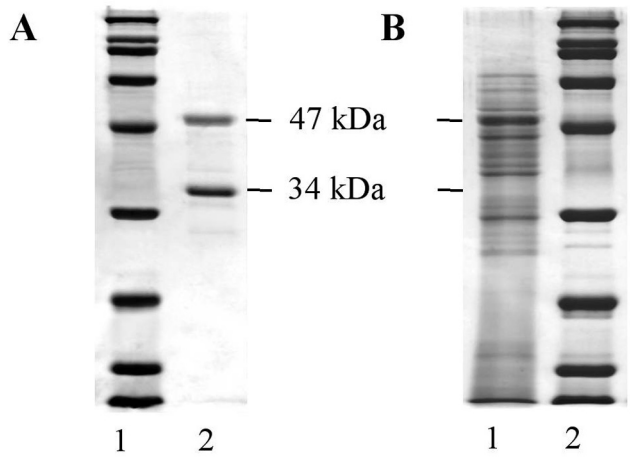


**FIG. 2.** SDS-PAGE analysis of *Chlorella* LOX. (A) LOX purified by sequential adsorption chromatography (lane 2) with M.W. markers (lane 1). (B) LOX extract resolved using size-exclusion chromatography on Sephacryl 200 (Bio-Rad, Hercules, CA) column (lane 1) with M.W. markers (lane 2). For abbreviation see Figure 1.

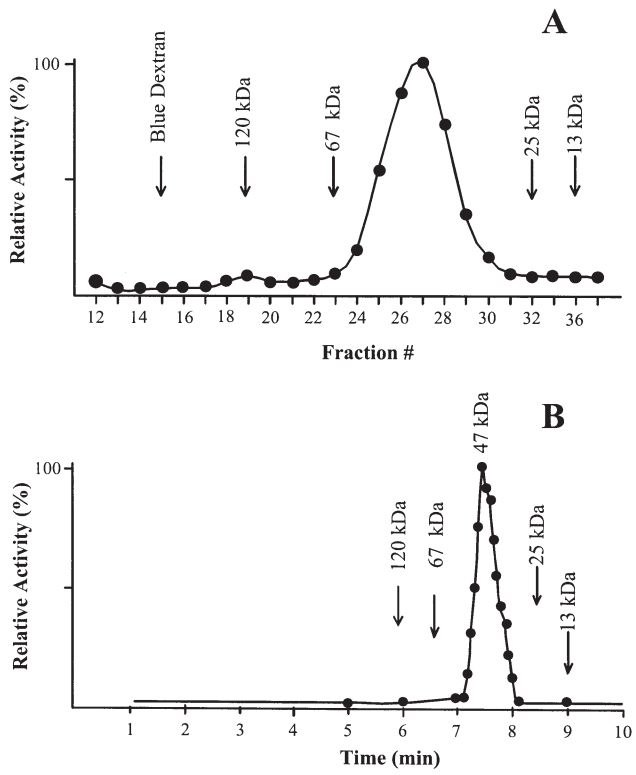


**FIG. 3.** LOX activity profile by native size exclusion chromatography (SEC) with molecular markers (↓). (A) Elution profile on Sephacryl S-200 column; (B) LOX activity profile (fractions 24–30, Panel A) on a Bio-Silect SEC 125-5 column (see the Materials and Methods section). For abbreviation and manufacturer, see Figures 1 and 2, respectively.

Although most LOX enzymes reported in higher plants, animals, and fungi are in the size range of 80–120 kDa, smaller proteins with LOX activity also have been reported. In potato tubers two LOX isozymes of 35 and 85 kDa size have been reported by Reddanna *et al.* (16). The alga *Ulva lactuca* also was reported to have two LOX isozymes with the major activity corresponding to a LOX protein of 41 kDa size and a minor protein with LOX activity of 116 kDa size (17). This later report is quite consistent with the size determined for the LOX isolated from *Chlorella* in this work.

Anaerobic cleavage of the 13-HPOD by the LOX enzyme also was associated with the purified 47 kDa enzyme, as determined by GC–MS analysis of the headspace volatiles, in accordance with our previous finding of anaerobic activity associated with LOX in *Chlorella* (10).

*Characterization of purified LOX products*. Recently we reported an LC/EI-MS method that allowed for separation and identification of the methyl esters of HPOD isomers from LA (13). Characteristic ion fragments for the HPOD isomers allowed the positional assignment of the hydroperoxide group in the HPOD isomers. In general, EI-MS of HPOD isomers does not yield a molecular ion but produces characteristic ions at *m/z* 310 [M – oxygen], 308 [M – $H_2O$], and 293 [M – $O_2H$]. An ion fragment at *m/z* 185 was observed exclusively for the 9-HPOD isomer, while 13-HPOD has a characteristic ion at *m/z* 99. Also, the 10-HPOD and 12-HPOD [12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid] isomers can be identified from fragment ions that are distinctive for these HPOD isomers. EI-MS analysis does not distinguish between the *Z,E* and *E,E* conjugated forms of the isomers; UV, however, provides evidence for these geometrical isomers since the *Z,E* and the *E,E* isomers have maximal absorptions at 233 and 228 nm, respectively (18).

The purified and concentrated active LOX fractions obtained by sequential adsorption chromatography were incu-

bated with LA under oxygen, and the reaction products were analyzed by LC/EI-MS after extraction and methylation. Figure 4A, the 232 nm UV chromatogram for these products, shows three peaks, which is consistent with three products having conjugated double bonds. The total ion current chromatogram (TIC) in Figure 4B shows the corresponding number of peaks. The EI spectra of the peaks gave characteristic ions for HPOD at *m/z* 310, 308, and 293. The peaks eluting at approximately 46 and 49 min (labeled I and II in Fig. 4B) were identified as the 9(*Z*),11(*E*) and 9(*E*),11(*E*)-13-HPOD isomers from the characteristic ion at *m/z* 99 and UV absorptions at 232 and 228 nm, respectively (13). The peak eluting at approximately 51 min (labeled III in Fig. 4B) had an ion at *m/z* 185 and UV absorption at 232 nm, characteristic of the 10(*E*),12(*Z*)-9-HPOD isomer. The small companion peak at 54 min (labeled IV in Fig. 4B) was identified as the 10(*E*),12(*E*)-9-HPOD. The product ratio for the 9- and 13 HPOD isomers was 49 and 51%, respectively. This product ratio also was obtained with the 35–80% $(NH_4)_2SO_4$ fractions (see below), indicating that the LOX activity recovered after absorption chromatography retained its initial activity.

Most LOX enzymes catalyze the formation of one particular regiospecific isomer with a stereospecific configuration *S* (1,2). The sterospecificity of the *Chlorella* products has not been reported, but the product distribution found in this study is similar to the product ratio produced from LA by LOX present in the blue-green microalga *Oscillatoria* sp. (6), where the LOX products from the microalga were concluded to be 13-(*S*)-HPOD and 9-(*S*)-HPOD in a ratio of 52:48.

*pH effect on product formation*. The ratio between the 9- and 13-HPOD isomers can be affected by reaction pH. For example, soybean LOX produces 13-HPOD almost exclusively at pH values above 8.5, whereas the 9-HPOD isomer is 25% of the total HPOD product at pH 6 (19). This pH dependency on product distribution is thought to be related to the orientation of the LA substrate at the active site. At the lowest pH, the protonated carboxylic acid group of LA can orient head-to-tail or tail-to-head at the active site, whereas at the higher pH the carboxylate anion can only be oriented one way (1,19). Since LOX from *Chlorella* produces both the 9- and 13-HPOD isomers, a pH dependency could be associated with HPOD product distribution. To investigate this aspect, the products isolated from the oxidation of LA by LOX present in the 35–80% $(NH_4)_2SO_4$ fraction of *Chlorella* extracts at pH 6, 7, 8, and 9 were characterized by LC/EI-MS. This analysis showed that the ratio between the 9- and 13-HPOD isomers is not significantly altered, remaining in a ratio close to 50:50, indicating that pH did not alter the product ratio.

In further pH studies, activity of the purified fractions from *Chlorella* showed that the LOX activity producing the 9- and 13-HPOD isomers is maximal at pH 7.5, which is consistent with the pH for optimal activity reported by Zimmerman and Vick (5).

In this study, we purified the LOX activity in *Chlorella* producing the 9- and 13-HPOD isomers. The LOX activity was derived from a monomeric enzyme with a molecular weight of 47 kDa, as determined by SDS-PAGE after sequential adsorption chromatography and native size determination by SEC. The product distribution ratio was not dependent on pH, and the 9- and 13- HPOD isomers were formed in an equal ratio by this LOX, which has optimal activity at pH 7.5.

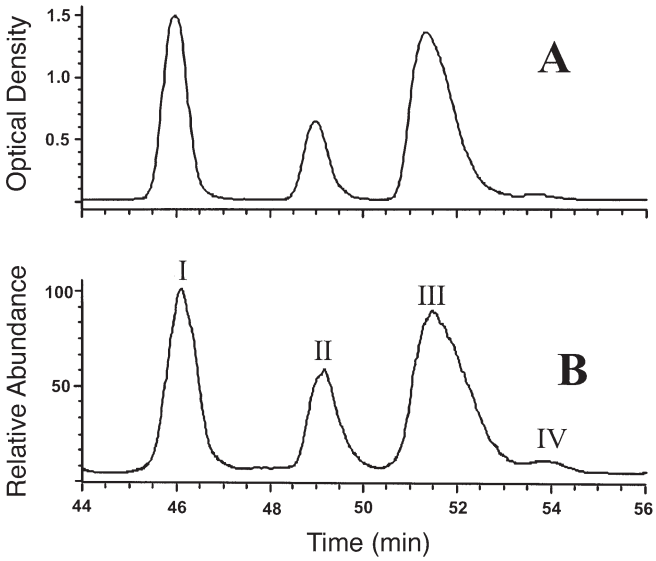


**FIG. 4.** LC/EI-MS analysis with a Diol column of the hydroperoxyoctadecadienoic acid products obtained by incubation of the chromatographically purified LOX activity with linoleic acid under oxygen. (A) UV chromatogram at 234 nm; (B) MS total ion chromatogram. Peak number, HPOD isomer: I, 9(*Z*),11(*E*)-13-HPOD; II, 9(*E*),11(*E*)-13-HPOD; III, 10(*E*),12(*Z*)-9-HPOD; and IV, 10(*E*),12(*E*)-9-HPOD.

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